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Short communication

An in-well direct lysis method for rapid detection of SARS-CoV-2 by real time RT-PCR in eSwab specimens

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ARTICLE INFO

Keywords:

Direct PCR

SARS-CoV-2

IGEPAL-CA-630

Crude lysate

ABSTRACT

Background: Diagnostic real time reverse transcription PCR (rRT-PCR) is usually done using nucleic acid (NA) purified from the sample. In the SARS-CoV-2 pandemic reagents and utensils for NA purification has been in short supply. This has generated interest in methods that eliminate the need for NA purification.

Objectives: To investigate if addition of detergent to rRT-PCR master mix (MM) enabled in-well direct lysis and detection of SARS-CoV-2 in clinical eSwab specimens.

Study design: IGEPAL-CA-630 (IGEPAL) was added to SARS-CoV-2 MM to 0.3 % final concentration and crude sample was added directly to the PCR well containing MM. Cycle of positivity (Cp) and categorical agreement was compared in samples tested in standard rRT-PCR after NA purification and in in-well lysis, direct rRT-PCR.

Results: In-well lysis direct rRT-PCR detected SARS-CoV-2 in 27/30 previously SARS-CoV-2+ samples with an average bias of 3.26 cycles (95 %CI: 0.08–6.43 cycles). All 30 previously test negative samples remained negative when tested in in-well lysis, direct PCR.

Conclusions: Supplementation of detergent to MM was shown to be useful for the detection of SARS CoV-2 in eSwab specimens (COPAN) by direct rRT-PCR without prior NA purification.

1. Background

Diagnostic PCR assays are usually performed using purified nucleic acid (NA) as template to ensure optimal performance. In the current SARS-CoV-2 pandemic commercial assays are few and limited in supply. To provide sufficient and timely diagnostic capabilities many laboratories have adapted various laboratory developed assays disseminated through the WHO website ([whoinhouseassays.pdf \[Internet\]](https://www.who.int/publications-detail/who-house-assays), 2021) to their available molecular workflows. A key platform component is automated liquid handlers capable of performing NA purification, e.g. MagNA Pure (Roche Diagnostics), Qiagen Symphony (QIAGEN), EMAG (bioMérieux). Supply of dedicated reagents and utensils for these liquid handlers is often less than demand and this has generated an interest for alternative methods for preparing samples for rRT-PCR.

Preparation of a crude lysate for PCR by boiling the sample is a

standard technique used in molecular biology (e.g. for colony PCR to screen for recombinants). Heat treatment of swab specimens as preparation for PCR for Herpes Simplex and Varicella Zoster Virus directly on clinical specimens resulted in only marginal loss of analytical sensitivity (Pandori et al., 2006; Buelow et al., 2013; Fan et al., 2014). Several recent reports (Fomsgaard and Rosenstjerne, 2020; Bruce et al., 2020) suggest that swab specimens could be prepared for SARS CoV-2 diagnostics by rRT-PCR by short heat treatment. Heat treatment, however, involves additional manipulation of sample, which increases risk of sample to sample contamination.

Direct lysis protocols are sometimes used in cellular gene expression studies. In a comparison study cell lysis using a hypotonic solution of BSA gave results comparable to standard spin column RNA purification (Svec et al., 2013). Non-ionic detergents sometimes in combination with BSA have been used for direct cellular lysis in preparation of cells for

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<https://doi.org/10.1016/j.jviromet.2021.114062>

Received 5 August 2020; Received in revised form 30 December 2020; Accepted 1 January 2021

Available online 8 January 2021

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rRT-PCR studies (Eaton et al., 1997; Le et al., 2015; Shatzkes et al., 2014).

2. Objectives

To investigate if addition of detergent to rRT-PCR master mix (MM) enabled in-well direct lysis and detection of SARS-CoV-2 in clinical eSwab specimens.

3. Study design

SARS-CoV-2 testing was done by rRT-PCR targeting the E-gene after purification of total NA (TNA) on a MagNA Pure 96 using the MagNA Pure 96 DNA and Viral NA small volume kit and 190 µl of sample material mixed with 10 µl of sample processing control. TNA was eluted in 100 µl. The primers and probes of the E-gene have previously been published (Corman et al., 2020), sample processing control was detected in a separate reaction. Final concentrations of E-gene specific primers and probes were 400 nM E_Sarbeco_F primer, 700 nM E_Sarbeco_R primer, 150 nM E_Sarbeco_P1 probe. Reactions were set up using 8 µl eluate and TaqMan Fast Virus 1-Step master mix (FV1S MM, Thermo Fischer Scientific) supplemented with 0.2 mM dUTP in a 20 µl reaction volume. PCR was performed on the LightCycler 480 instrument in 384 well plates with the following PCR profile: 5 min of 50 °C and 20 s of 95 °C followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

For initial experiments direct lysis were performed using the E-gene targeted rRT-PCR described above and differing concentrations of IGEPAL CA-630 (Sigma-Aldrich) and bovine serum albumin (BSA, Sigma-Aldrich). Then the PCR was transferred to a 96-well format using 5 µl E-swab inoculum in a 50 µl reaction volume and optimized primers and probe concentrations. For FV1S MM the 100 nM E_Sarbeco_F primer, 400 nM E_Sarbeco_R primer, 150 nM E_Sarbeco_P1 probe was used; for Luna Universal Probe One-Step RT-qPCR MM (Luna MM) 100 nM E_Sarbeco_F primer, 700 nM E_Sarbeco_R primer, 200 nM E_Sarbeco_P1 probe was used. PCR cycling conditions were unchanged from the standard SARS-CoV-2 rRT-PCR in 384 well format.

4. Results

We first titrated IGEPAL (to final concentrations 0 %, 0.2 %, and 0.4 %) and BSA (to final concentrations 0 %, 0.8 %, and 1.6 %) in check-board fashion in SARS-CoV-2 rRT-PCR assay directed against the E-gene and using FV1S MM using two SARS-CoV-2-positive eSwab specimens. Addition of IGEPAL decreased Cp by 6.1 cycles and 2.2 cycles for sample B and D, respectively (Fig. 1) with no apparent effect of increasing the concentration of IGEPAL. Only minute, non-directional effects of BSA were observed. Purified TNA from two SARS-CoV-2+

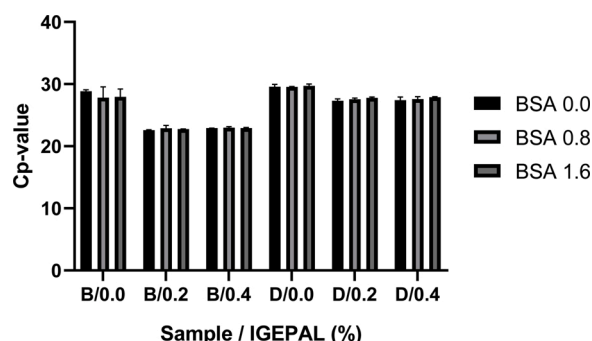


Fig. 1. Two clinical eSwab samples, B and D, were inoculated in duplicate into master mixes containing the indicated concentrations of IGEPAL CA-630 and BSA. For both samples a reduction in Cp-value was obtained upon addition of IGEPAL CA-630. Only minimal and non-directional effects of addition of BSA were observed.

samples were tested again in MM containing 0.0, 0.2, and 0.4 % IGEPAL to investigate if IGEPAL inhibited PCR. Addition of IGEPAL increased average Cp with 0.22/0.27 cycles and 0.13/0.08 cycles at 0.2 % and 0.4 % IGEPAL for the two samples. For further experiments, we decided to use IGEPAL at a final concentration of 0.3 % and not to include BSA.

We next investigated if eSwab medium influenced rRT-PCR. MagNA Pure purified TNA from a SARS-CoV-2-positive sample was diluted in uninoculated eSwab medium to simulate different sample volumes added to the reaction mix using 6 replicates (Table 1). Addition of eSwab medium to the reaction mix inhibited rRT-PCR (ANOVA $P < 0.001$); proportions corresponding to 10 % of reaction volume resulted in a small, but significant 0.17 cycle increase in mean Cp-value further increasing to 2.50 cycles at 36 % of reaction volume. As further inhibition from substances present in directly lysed clinical swab material should be expected, we decided to limit the volume of eSwab sample material to 10 % of reaction volume.

In order to preserve analytical sensitivity, we adapted the SARS-CoV-2 rRT-PCR to a 50 µl reaction volume in a 96 well plate. Using 5 µl of sample eSwab in the direct lysis protocol we analyzed 30 SARS-CoV-2+ samples and 30 SARS-CoV-2- samples. The direct lysis method detected SARS-CoV-2 in 27/30 previously positive samples (sensitivity 0.90; 95 %CI: 0.74–0.96). The three undetected samples all had Cp >35 in our standard assay. All 30/30 negative samples were also undetected in the direct lysis assay. Samples detected in both assays displayed an average bias in Cp of 3.26 cycles (95 %CI: 0.08–6.43 cycles) (cf. Fig. 2A). Of note, Cp increased between 0.12 and 8.04 cycles using direct lysis (Fig. 2A). Comparable results were obtained with Luna MM with 27/30 positive samples detected with a larger average bias in Cp of 4.96 (95 %CI: 2.10–7.81) (Fig. 2B), however, 1/10 previously negative samples was positive with a Cp-value of 38.78.

Repeated experiments using the same samples and different sample volumes indicated that the increase in Cp-values was sample specific. To investigate if the Cp increase was caused by inhibitors, we spiked 10 SARS-CoV-2+ and 10 negative samples with Phocine Distemper Virus (PDV) and detected SARS-CoV-2 and PDV in separate reactions. PDV was uniformly detected in all samples (SD: 0.54 cycles) and PDV Cps did not correlate with difference in detection of SARS-CoV-2 after direct lysis and NA purification.

5. Discussion

Here we show that a direct in-well lysis method can detect SARS-CoV-2 by rRT-PCR with an estimated 90 % sensitivity and an average loss of 3.3 cycles in Cp compared to automated NA purification prior to rRT-PCR. This is comparable with the reported loss of sensitivity when using heat lysis of samples prior to rRT-PCR (Fomsgaard and Rosenstjerne, 2020; Bruce et al., 2020), but with a simpler workflow. We did not test the direct lysis method with commercial SARS-CoV-2 PCR assays. Previously, it was reported that the RealStar SARS-CoV-2 Assay was susceptible to inhibitors present in crude lysates of clinical swab samples (Fomsgaard and Rosenstjerne, 2020), and direct lysis therefore needs validation with the specific assay employed.

NA purification delivers a larger fraction of original sample to PCR explaining up to 1.6 cycle difference in Cp in our setting. Samples varied in the loss of Cp cycles compared to NA purification. If exogenous RNA

Table 1
Inhibition of SARS-CoV-2 rRT-PCR detection by eSwab medium.

eSwab proportion of total medium	Mean Cp (N=6) (95% CI)	Cp difference (X-A) (95% CI)
A: 0 %	28.50 (28.34–28.66)	ref.
B: 10 %	28.67 (28.62–28.73)	0.17 (0.03–0.32)
C: 20 %	28.84 (28.66–29.03)	0.34 (0.14–0.55)
D: 30 %	29.17 (28.81–29.52)	0.67 (0.33–1.00)
E: 36 %	31.00 (30.68–31.32)	2.50 (2.19–2.81)

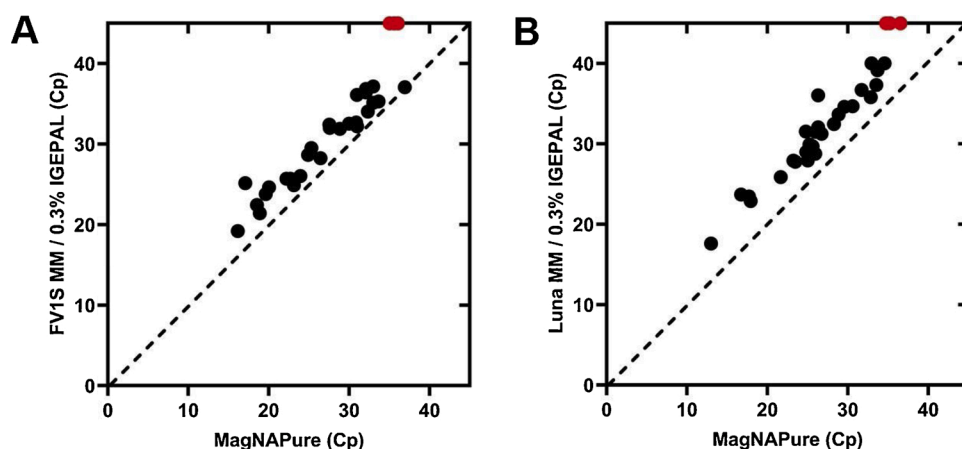


Fig. 2. Comparison between standard PCR after NA purification and direct, in-well lysis PCR using either FV1S MM (A) or Luna MM (B). Thirty eSwab samples that tested positive in standard PCR was tested again in direct, in-well lysis PCR and the resulting Cps are shown. Samples not detected by direct PCR, but by standard PCR are indicated with red dots and arbitrarily assigned a Cp-value 45. Thirty previously negative samples were also unreactive in direct, in-well lysis PCR using FV1S, however, 1/10 previously negative was reactive when tested in Luna MM. Two different sets of samples were used to obtain the data presented in (A) and (B).

virus was spiked into the sample Cps did not vary arguing against PCR inhibition or RNA degradation. A likely explanation is that in some samples SARS-CoV-2 RNA is less accessible after detergent treatment. Some commercial cell lysis agents include a protease in the lysis step. This may decrease variation between samples, but necessitates an additional step to stop lysis before adding sample to MM.

We examined samples collected into eSwabs reflecting our current diagnostic challenge. This transport medium may inhibit rRT-PCR. Dry swabs allow the laboratory to optimize lysis buffer for analytical performance. A recent study reported favorable results eluting dry swabs in Tris-EDTA and directly performing rRT-PCR on the eluate (Srivatsan et al., 2020). Dry swabs are also amenable to enzymatic lysis buffers that may be inactivated prior to rRT-PCR. However, this flexibility comes at the expense of the in-well workflow described here.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Rikke Lind Jørgensen: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Martin Schou Pedersen:** Conceptualization, Visualization, Writing - review & editing. **Alisha Shazad Chauhan:** Investigation. **Louise Munkholm Andreasson:** Investigation. **Gitte Qvist Kristiansen:** Conceptualization, Methodology, Writing - review & editing. **Jan Gorm Lisby:** Conceptualization, Supervision, Writing - review & editing. **Maiken Worsøe Rosenstjerne:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Kristian Schønning:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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